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In vitro antioxidant activity of *Aplysia depilans* ink colleted from Bizerte Channel (NE Tunisia)

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Abstract: The ink secretion of molluscan species was identified as one of the novel sources of bioactive compounds. The present study aims to evaluate the *in vitro* antioxidant activity of *Aplysia depilans* ink extract. The antioxidant activity of ink extract were evaluated using 2,2- diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, Hydroxyl radical scavenging activity, Ferric ion reducing power (FRP) and Ferrous ion chelating (FIC) activity. The results from the present work revealed the strongest antioxidant activity of *Aplysia depilans* ink. The electrophoretic profile showed band with molecular weight of 60 kDa. The highest antioxidant activity in ink extract probably may be due to the presence of this protein with lower moleculair weight.
Keywords: *antioxidant activity; cephalopods; ink extract; Aplysia fasciata; sea hare*

I. Introduction

Lipid oxidation causes some health hazards in human beings such as cardiovascular disease, cancer, and neurological disorders as well as aging process [1-2]. To prevent or slow down lipid oxidation, several antioxidants including synthetic and natural antioxydants have been widely used. However, synthetic antioxydants are suspected of being toxic upon long-term exposure [3]. As a consequence, natural antioxidants have gained increasing attention. In the past few decades, mining of bioactive compounds from marine sources are considered promising because of its rich species diversity [4]. Earlier studies by various researchers reported that molluscs have good antioxydant properties [5-6]. Several natural bioactive compounds like peptides, sterols, terpenes, polypropionates, nitrogenous compounds, prostaglandins, fatty acid derivatives, miscellaneous compounds and alkaloids were reported from molluscs which were identified as essential with specific types of activities [7]. Cephalopod ink has been proved to be an alternative medicine and has a wide range of therapeutic applications [8].
On the basis of its traditional use and literature reference, the present study focuses the antioxydant activity of *Aplysia depilans* ink extracts by different methods.

II. Materials and methods

II.1. Collection of ink samples

30 adult specimens of the sea hare, (*Aplysia depilans,* Gmelin, 1791), ranging in length from 20-25 cm, were collected during low tides from the Bizerte Channel, Tunisia (37°8' and 37°14' N, 9°46' and 9°56' E). The Channel represents a transition area between Bizerte Bay and Bizerte Lagoon (fig 1). The animals were transported to the laboratory in a container with sea water. The ink fluid was obtained by disturbing the animals and extracted with water. All aqueous ink samples were centrifuged at 15,000 g for 15 min as described by [9] and the supernatant was taken and lyophilized to a black residue using a lyophilizer and stored for further use.

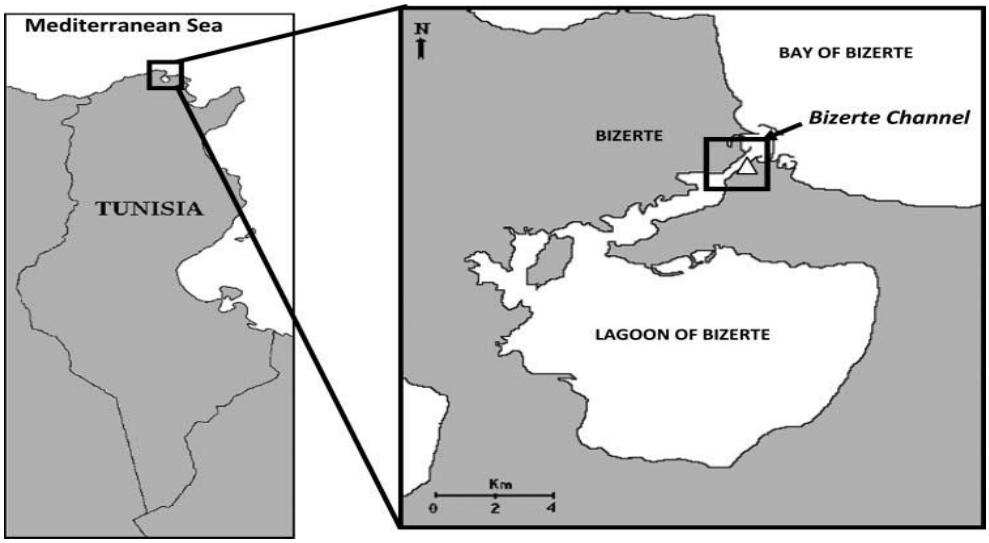

Figure 1: Map of the Bizerte Lagoon showing the sampling site (Channel) from where sea hare (Aplysia depilans, Gmelin, 1791) were collected.

II.2. Estimation of protein concentration

Protein concentration was determined by the method of Bradford [10] using bovine serum albumin (BSA) as a standard.

II.3. Chemicals

All chemicals and solvents were purchased from Sigma-Aldrich, and they were of highest purity and analytical grade.

II.4. Determination of *in vitro* antioxidative activities

The extract of *Aplysia depilans* ink was tested for antioxidative activities by 2,2,-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, Hydroxyl Radical Scavenging Activity, Ferric Reducing Antioxydant Power (FRAP) and Ferrous Ion Chelating Activity (FIC).

II.5. DPPH radical scavenging activity.

The DPPH radical scavenging assay employed is as described by [11]. Various dilutions of the methanolic solution and standard (ascorbic acid) (0.003-0.3 mg/mL, in triplicate) were added to DPPH solution (0.035 mg/mL). The mixture was left in the dark for 30 min before reading the absorbance at 517 nm with methanol as blank. The control consisted of methanol in place of sample. Radical scavenging activity was expressed as a percentage and calculated using the formula:
% Scavenging = ((Abs. control - Abs. sample) / Abs. control) * 100.
Where Abs control is the absorbance of the control reaction and Abs sample is the absorbance in the presence of the sample.
Result was presented as IC_{50} the concentration of the substrate which causes an inhibition of 50% of the activity of DPPH.

II.6. Hydroxyl radical-scavenging activity

The hydroxyl radical scavenging activity was measured by the desoxyribose method [12] and compared with ascorbic acid. It was carried out by measuring the competition between desoxyribose and the compounds that generate hydroxyl radicals from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radicals on desoxyribose led to formation of thiobarbituric acid-reactive substances (TBARS) which were measured by the method of Ohkawa *et al,* [13]. The hydroxyl radical is the most reactive oxygen species (ROS) that attacks almost every molecule in the body and also leads to DNA damage in a cell. It initiates the peroxidation of cell membrane lipids increases MDA levels which is cytotoxic, mutagenic and carcinogenic. Results was expressed as a percentage and calculated using the formula:
(%) OH scavenging = ((Abs control - Abs sample) / Abs control) * 100
Where Abs control is the absorbance of the control reaction and Abs sample is the absorbance in the presence of the sample.

II.7. Ferric ion reducing antioxidant power (FRAP).

The reducing power of methanolic solution was determined according to the method of Oyaizu [14] and compared with ascorbic acid. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. One mL of test sample solution or standart at various concentrations (0.003-0.3 mg/mL) was mixed with phosphate buffer (0.2 M) and potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (10 %) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water and a freshly prepared ferric chloride solution (0.1 %). The absorbance was measured at 700 nm. Ascorbic acid was used as standard. A blank was prepared without adding standard or test compound. Increased absorbance of the reaction mixture indicates increase in reducing power. The percent increase in reducing power was calculated using the following equation:
((Abs test – Abs blank) / Abs blank) * 100
Where Abs test is absorbance of test solution; Abs blank is absorbance of the blank.
IC_{50} corresponds to the concentration necessary to reduce 50 % of ferric ferrous complex. The lower value indicates a higher reducing power. IC_{50} is expressed in mg/mL for the pure compounds.

II.8. Ferrous ion chelating activity (FIC)

The chelating of ferrous ion by the methanol sample solution was estimated by the method of Singh and Rajini [15]. Various dilutions of the metanolic solution and standard (in triplicate) were added to $FeSO_4$ (0.1 mM) and ferrozine (0.25 mM). The tubes were shaken well and left to stand for 10 min. The absorbance was measured at 562 nm, against blank containing water in place of ferrozine. The control consisted of water in place of the sample. The ability of a sample to chelate ferrous ion was calculated as follows:

Chelating effect (%) = ((Abs. control – Abs. sample) / Abs control) * 100

IC50 corresponds to the concentration necessary to prevent 50 % from initial Fe^{2+}-ferrozine complex formation. The lower value indicates that the compound is more chelator. IC_{50} is expressed in mg/mL for the pure compounds.

II.9. SDS-Polyacrylamide gel electrophoresis

Electrophoresis of the crude ink was carried out by the method of Laemmli [16] on a 1-mm vertical gel consisted of 5% stacking gel mix, and main running gel mix of 12.0% acrylamide. Ink samples containing 2% SDS and 1% 2-mercaptoethanol were incubated at 100°C for 10 min. A few sucrose crystals were dissolved in the samples before being applied (30 µL) to the gel. Electrophoresis was carried out at 20-mA constant current for 60 min and protein bands were visualized by staining in

Coomasie brilliant blue. Standard molecular weight markers sizes ranging from 35 to 225 kDa were used to determine the molecular weight of individual proteins.

II.10. Statistical analysis

IC_{50} values were calculated by linear regression. Means ± SD were calculated. The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. P values less than 0.05 were considered significant. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

III. Results

III. 1. DPPH radical scavenging activity

DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants [14]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [17]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid is the reagents used as standards. The sample is able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. *Aplysia depilans* ink is likely to have the effect of scavenging free radical with an IC_{50} = 0.94 mg/mL when compared with ascorbic acid IC_{50} = 0.0396 mg/mL (Fig 2). The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases [18]. It has been found that ascorbic acid reduce and decolorize DPPH by his hydrogen donating ability [19], aplysia extract is probably involved in their antiradical activity.

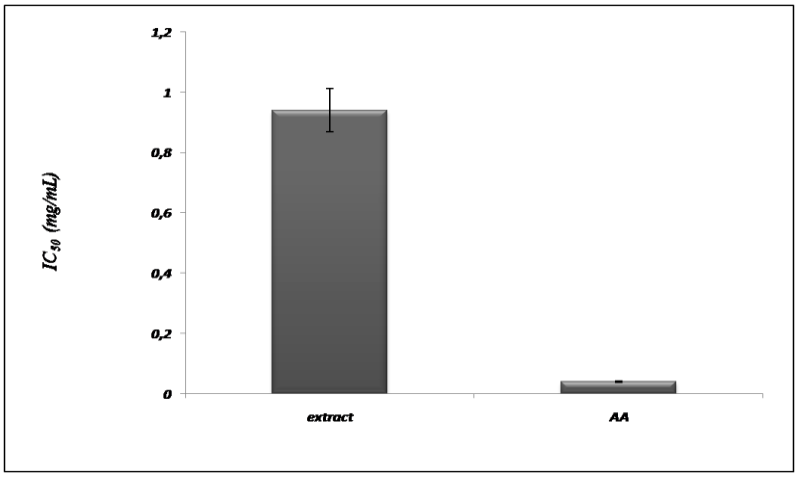

Figure 2: DPPH radical scavenging activity, (Extract: Aplysia ink extract, AA : ascorbic acid)

III.2. Hydroxyl radical-scavenging activity

The hydroxyl radical is the most reactive oxygen species (ROS) that attacks almost every molecule in the body and also leads to DNA damage in a cell. It initiates the peroxidation of cell membrane lipids [20-21], increases MDA levels which are cytotoxic, mutagenic and carcinogenic [22]. Aplysia ink extract showed significant hydroxyl radical scavenging activity with an IC_{50} of 7.43 µg/ml when compared with ascorbic acid IC_{50}: 6.24 µg/mL and gallic acid IC_{50}: 2.17 µg/mL

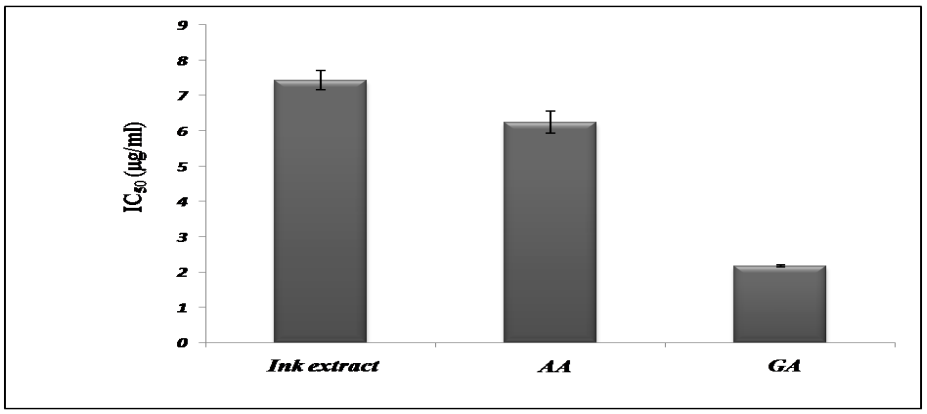

Figure 3: Hydroxyl radical scavenging activity in (%) (Extract: Aplysia ink extract, AA : ascorbic acid; GA gallic acid).

III.3. Reducing power determination

It has been observed a direct correlation between antioxidant activity and reducing power of certain compound. Figure 4 shows the reductive capability of aplysia ink compared to ascorbic acid. For the measurements of the reductive ability, it has been investigated from the Fe^{3+}–Fe^{2+} transformation in the presence of compounds using the method followed by Oyaizu [14]. The extract exhibited reducing power activity with an IC_{50} at 39.4 µg/mL wich is lower than that of ascorbic acid IC_{50} : 3.56 µg/mL.

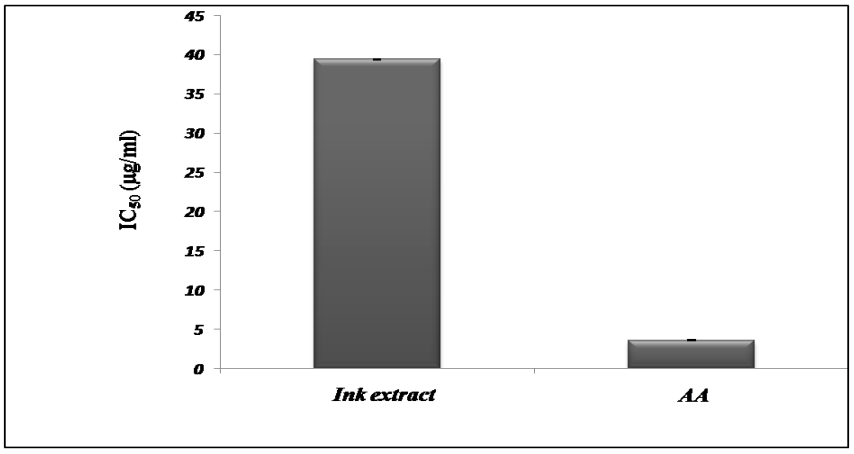

Figure 4: Reducing power activity (Ink extract: Aplysia ink extract, AA : ascorbic acid)

III.4. Ferrous ion chelating (FIC) activity.

The method of ferrous ion chelating activity is based on chelating of Fe^{2+} ions by the reagent ferrozine, which is a quantitative formation of a complex with Fe^{2+} ions [23]. The formation of a complex is probably disturbed by the other chelating reagents, which would result in the reduction of the formation of violet-colored complex. Measurement of the rate of reduction of the color, therefore, allows estimation of the chelating activity of the coexisting chelators. In this assay both compound and standard compound interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating activity and captures the ferrous ion before ferrozine. The ferrous ion chelating effect of the aplysia ink showed an IC_{50} at 2.31 µg/ml compared with ascorbic acid (IC_{50}: 5.77 µg/mL). Metal chelating capacity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation [24]. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby, stabilizing the oxidised form of the metal ion [25]. The data obtained from Figure 5 reveal aplysia ink demonstrate a high

capacity for iron binding more than standard, suggesting that its action as antioxidant may be related to its iron-binding capacity.

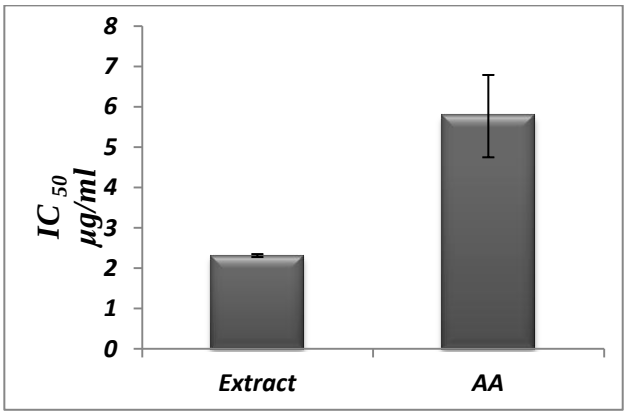

Figure 5: Iron chelating activity (Extract: Aplysia ink extract, AA: ascorbic acid)

III.5. SDS-PAGE analysis

The electrophoretic profile of the ink sample showed the presence of protein with molecular weight of 60 kDa.

IV. Discussion

Marine mollusks are protecting themselves from predators through their unique way; one of them is releasing ink when disturbed. This ink secretion contains a rich array of chemical secretions to escape from predators. The chemical composition of the ink of all inking mollusk will not be the same. In this study we have accessed antioxidant activity of *Aplysia depilans* ink samples using several assays as shown in fig. 2, 3 and 4.

Ink extract was effective in scavenging DPPH radicals. DPPH radical scavenging activity was based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the non-radical species [26- 27]. DPPH is a radical having an odd electron and reacts with hydrogen donated from antioxidant.

The ink extract exhibited reducing power activity with an IC_{50} = 39.4 µg/mL wich is lower than that of ascorbic acid IC_{50} = 3.56 µg/mL. The ferrous ion chelating effect showed an IC_{50} = 2.31 µg/ml compared with ascorbic acid (IC_{50} = 5.77 µg/mL). Ferric reducing power activity is generally used to measure the capacity of a substance in reducing TPTZ-Fe (III) complex to TPTZ-Fe(II) complex [28-26]. The result indicated that ink extract was able to act as reducing agent which provided electron for stabilization. Additionally, some compounds in the ink could chelate prooxidative metals, thereby lowering or retarding the initiation of lipid oxidation process. Ink from *Sepia officinalis* and *Loligo formosana* was reported to function as antioxidant [29- 30].

The results from the present work revealed the strongest antioxidant activity of sea hare ink. The highest antioxidant activity in ink extract probably may be due to the presence of peptides or proteins with lower moleculair weight [31]. SDS polyacrylamide gel electrophoresis of aqueous extracts of ink samples showed the presence of proteins with the molecular weight of 60 kDa. This protein may be responsible for the antioxydative activitie of the ink samples. This result supports the previous work done by Xin Guo *et al.* [32] hwo reported that proteins isolated from squid ink with molecular weight of 10-50 kDa and more, showed higher antioxidant activity than other fractions. SDS polyacrylamide gel electrophoresis of aqueous extracts of ink samples of some molluscan showed the presence of proteins with the molecular weight ranging from 62 to 249 kDa. These proteins may be responsible for various biological activities of the ink samples [9]. Also, Rajaganapathi *et al*, [33] has purified from the purple ink of the sea hare *Bursatella leachii* a protein with 60-kDa moleculair weight, that showing anti-HIV activity.

V. Conclusion

In conclusion, our results showed that *Aplysia depilans* ink as a natural compound is a new free radical scavenger with a promising prospect.

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